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Abstract

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DEOXYURIDINE SUPPRESSION TEST: A COMPARISON OF TWO METHODS AND EFFECT OF THYMIDINE ON THE INCORPORATION OF ^{3}H-DEOXYURIDINE INTO DNA IN HUMAN BONE MARROW CELLS

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Abstract. Deoxyuridine suppression tests have been performed by two different methods on six normoblastic and eight megaloblastic marrows. A good correlation was obtained between the results by the modified and the original methods. The simplified method was found to be applicable for a clinical purpose to diagnose megaloblastosis in the marrow. Uptake of ^{3}H-deoxyuridine into DNA and effect of various concentrations of thymidine was studied on five normoblastic and six megaloblastic marrows. In megaloblastic marrows, a greater amount of thymidine was required to obtain the same rate of suppression of ^{3}H-deoxyuridine incorporation into DNA than in normoblastic marrows. Impairment of thymidine incorporation into DNA in megaloblastic marrows was not revealed. Therefore, lower rate of suppression of ^{3}H-deoxyuridine by thymidine in megaloblastic marrows may be due to impairment of the incorporation of deoxyuridine before the addition of thymidine.

Deoxyuridine suppression (DUS) test has been proved to be of great value as a diagnostic tool of megaloblastic bone marrow. The interrelated biochemical pathways are shown in Fig. 1. Killman (1) introduced this test first using autoradiographic techniques. Metz *et al.* (2) modified the method for extraction of DNA. Several modifications have been proposed since then (3–6). Each of these modified methods is still too far complicated from clinical use. We have tried to modify the method as simply as possible and compared the result obtained by the new method with the method of Metz *et al.* (2). We also studied the effect of preincubation of marrow cells with cold thymidine (TdR) on the incorporation of ^{3}H-deoxyuridine (UdR) to elucidate a biochemical basis of this test.

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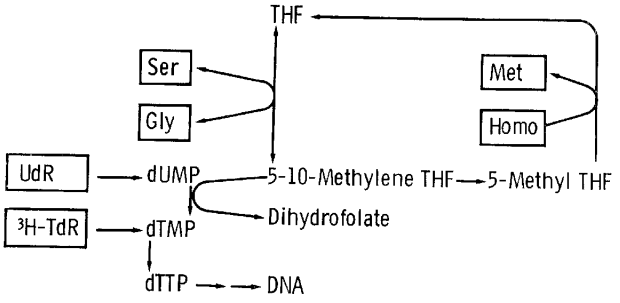

Fig. 1. Synthetic pathway of thymine moiety of DNA from deoxyuridine. THF=tetrahydrofolic acid, UdR=deoxyuridine, TdR=thymidine, dUMP=deoxyuridine monophosphate, dTMP=deoxythymidine monophosphate, dTTP=deoxythymidine triphosphate, Met=methionine, Homo=homocysteine, Ser=serine, Gly=glycine. Vitamin B_{12} is required when THF is formed from 5-methyl THF coupled with methionine synthesis.

MATERIALS AND METHODS

Bone marrow (3–5 ml) aspirated from the sternal bone or the anterior iliac crest was immediately mixed in a universal tube containing 10 ml of cold Hanks' balanced salt solution (HBSS) (pH 7.4) with 100 units/ml preservative free heparin. At the same time, venous blood was taken for the autologous serum. The samples were forced through a 25-gauge needle twice to break down cell clumps. The cells were washed twice with 10 ml of cold HBSS and then suspended in a mixture consisting of one part of autologous serum and 2 parts of HBSS. Cell counts were adjusted to 6,000–24,000 nucleated cells/mm^3. The volume of serum-HBSS solution added was usually about two times volume of washed bone marrow cells. DUS test was performed on half of this cell suspension by the method described by Metz *et al.* (2). Another half of the cell suspension was used for the modified method as follows. To 0.5 ml of the marrow cell suspension was added 0.4 ml of HBSS (Control) or 0.3 ml of HBSS with 0.1 ml of 2'-deoxyuridine (1 μ mole/ml)(Test). Both Control and Test tubes were set up in triplicate. The tubes were incubated for 1 hr at 37°C by shaking these tubes gently (90 cycle/min) on a metabolic shaker. After the incubation, 0.1 ml of ^{3}H-TdR solution (5 μCi/ml, specific activity: 23–25.6 Ci/mmole) was added to each tube. The tubes were incubated further at 37°C for 3 hr, then washed three times in cold 0.9% NaCl solution. After mixing well, two 0.1 ml aliquots of each cell suspension were delivered to each Whatman filter discs (1.9 cm in diameter and 3 mm in grade). The discs were dried overnight, immersed in a large volume (200 ml) of ice cold 10% trichloroacetic acid for 20 min at 4°C. The discs were placed into two changes of 100 ml volume of absolute methanol for 10 min each. After a brief rinse in acetone, the discs were dried and added with liquid scintillator solution (10 ml of toluene containing 0.3% 2, 5-diphenyloxazole and 0.03% p-bis (2-1, 5 phenyloxazolylbenzene)). The radioactivities on the discs were determined using a liquid scintillation counter (LKB-Wallac). Quenching was corrected by the external standard method. Results were ex-

pressed as a percentage of the counts on the discs from the tubes incubated with UdR (Test) to those without UdR (Control).

Incorporation of ^{3}H-UdR into DNA and effect of preincubation of the bone marrow cells with TdR (10^{-1}-10^{-5} μmole) were examined using the method of Metz *et al.* (2). Marrow cell suspension (0.3 ml) was added with 0.1 ml of TdR solution (1-10^{-4} μmole/ml in HBSS) and 0.5 ml of HBSS. Triplicate tubes were incubated for 1 hr at 37°C. As a control, triplicate tubes containing 0.3 ml of marrow cell suspension and 0.6 ml of HBSS were also incubated. After incubation, 0.1 ml of ^{3}H-UdR (10 μCi/ml, specific activity : 21-27 Ci/mmole) was added to each tube. After further incubation for 3 hr, cells were washed and DNA was extracted as described by Metz *et al.* (2). Suppression of the incorporation of ^{3}H-UdR by TdR was expressed as the percentage of the counts of the tubes with TdR to those without TdR. DUS test was performed on the same marrows at the same time. Absolute values of the ^{3}H-TdR and ^{3}H-UdR which were incorporated into DNA were calculated after converting radioactivity into disintegration per minute (dpm) by the use of the external standard method. Dpm of the standard ^{3}H-TdR and ^{3}H-UdR solutions (0.01 ml) were also counted. Absolute amount of ^{3}H-TdR and ^{3}H-UdR was expressed as μmoles/μg DNA and μmoles/10^{6} nucleated cells.

RESULTS

Two DUS methods were compared on six normoblastic and eight megaloblastic marrows. A good correlation was observed between results by the modified method and that of Metz *et al.* (2) (Table 1). In this method, all normoblastic marrows showed DUS value below 10% (range: 0.2-10%). In contrast, the levels in megaloblastic marrows were more than 10% (range: 13.7-66.5%). As many reports concluded, DUS level below 10% should be regarded as normal value.

Effects of various concentrations of TdR on the incorporation of ^{3}H-UdR were shown in Fig. 2. Inhibition of the incorporation of ^{3}H-UdR was observed as TdR concentration increased. A significant difference of the inhibitory effect of TdR was observed between normoblastic and megaloblastic marrows by adding 10^{-3} and 10^{-4} μmole of TdR. In normoblastic marrows, TdR suppressed the incorporation of ^{3}H-UdR to a larger extent than in megaloblastic marrows. Absolute amount of ^{3}H-TdR and ^{3}H-UdR incorporated into DNA and effect of each UdR and TdR were shown in Tables 2 and 3. In normoblastic marrows (five cases), ^{3}H-TdR uptake was suppressed by the addition of UdR (0.1 μmole) from 29.5×10^{-8} μmole/μg DNA and 37.1×10^{-7} μmole/10^{6} cells to 1.8 and 2.5, respectively. In megaloblastic marrows (six cases), suppression by UdR was from 26.2×10^{-8} μmole/μg DNA and 22.6×10^{-7} μmole/10^{6} cells to 8.3 and 10.1, respectively. Effects of 10^{-3} μmole of TdR on the incorporation of ^{3}H-UdR were as follows: in normoblastic marrows, from initial amounts of 12.8×10^{-8}

TABLE 1. HEMATOLOGICAL DATA AND RESULTS OF DEOXYURIDINE SUPPRESSION (DUS) TESTS

Case no.	Diagnosis	Bone marrow	Hb (g/dl)	MCV (fl)	Serum folate (μg/l)	Red cell folate (μg/l)	Serum V. B_{12} (ng/l)	DUS by Metz *et al.* (%)	DUS by modified method (%)
1	Iron deficiency	Normoblastic			3.8	600	145	8.8	10.0
2	ITP	„	9.1	90	13.2	375	135	2.7	0.2
3	Pregnancy	„	11.3	97	20	627	210	2.8	4.0
4	Coronary heart disease	„	15.8	82				5.5	6.0
5	Pregnancy	„	8.8	96		738	718	3.0	1.5
6	Liver cirrhosis	„	11.0	115	1.4	134	525	4.8	4.4
7	Pernicious anemia	Megaloblastic	10.7	87	7.4		65	16.5	13.7
8	Folate deficiency	„	8.6	85	0.4	78	120	51.0	43.6
9	Vegetarian, V. B_{12} def.	„	11.5	104	2.4		90	29.0	29.7
10	Pernicious anemia	„	13.1	114	12.8	134		51.7	49.6
11	„	„	12.9	98	9.2		38	47.7	53.6
12	„	„	7.3	97				50.6	66.5
13	Folic acid deficiency	„	11.5	97	2.3	102	100	42.4	52.3
14	Pernicious anemia	„	10.2	117	13.7	199	38	32.9	39.3
	Normal values		12.5–17	80–92	2–15	145–450	170–1000	<10	<10

Table 2. Uptake of ^{3}H-TdR and ^{3}H-UdR in normoblastic marrow cells and effects of preincubation with UdR (10^{-1} μmole) and TdR (10^{-3} μmole) respectively

Case no.	DUS test (%)	^{3}H-TdR uptake per DNA ($\times 10^{-8}$ μmole/μg DNA)	^{3}H-TdR uptake per cell ($\times 10^{-7}$ μmole/10^6 cells)	^{3}H-UdR uptake per DNA ($\times 10^{-8}$ μmole/μg DNA)	^{3}H-UdR uptake per cell ($\times 10^{-7}$ μmole/10^6 cells)
15	3.8	38.8–1.5	68.3–3.2 (4.7%)	9.2–1.6 (18.0%)	24.4–2.4 (9.9%)
16	8.3	14.5–1.2	20.0–1.2 (6.2%)	4.4–1.0 (22.4%)	12.5–2.5 (19.6%)
17	7.0	21.0–1.5	24.7–2.4 (9.7%)	14.3–1.9 (13.0%)	24.9–3.4 (13.5%)
18	9.2	33.6–3.1	34.0–2.8 (8.1%)	20.8–5.8 (27.8%)	21.0–5.1 (24.3%)
19	4.1	39.4–1.6	38.7–2.7 (6.9%)	15.2–1.8 (11.8%)	17.3–1.8 (10.2%)
Mean	6.5	29.5–1.8	37.1–2.5 (7.1%)	12.8–2.4 (18.6%)	20.0–3.0 (15.5%)
S.E.	1.09	5.0 0.3	8.5 0.3	2.8 0.9	2.3 0.6

Left part of each column shows the absolute amount of ^{3}H-TdR or ^{3}H-UdR without addition of UdR or TdR respectively. Right part of each column shows the amount after addition of UdR or TdR respectively. In brackets, percentage of the amount of ^{3}H-TdR or ^{3}H-UdR after addition of UdR or TdR respectively. S.E. =Standard error of the mean.

Table 3. Uptake of ^{3}H-TdR and ^{3}H-UdR in megaloblastic marrow cells and effects of preincubation with UdR (10^{-1} μmole) and TdR (10^{-3} μmole) respectively

Case no.	DUS test (%)	^{3}H-TdR uptake per DNA ($\times 10^{-8}$ μmole/μg DNA)	^{3}H-TdR uptake per cell ($\times 10^{-7}$ μmole/10^6 cells)	^{3}H-UdR uptake per DNA ($\times 10^{-8}$ μmole/μg DNA)	^{3}H-UdR uptake per cell ($\times 10^{-7}$ μmole/10^6 cells)
20	56.2	5.3– 3.0	8.5– 3.7 (43.3%)	2.3–1.1 (45.7%)	4.4–1.4 (31.8%)
21	63.7	23.4–14.9	5.6– 3.0 (55.0%)	5.7–3.5 (61.8%)	0.8–0.5 (70.5%)
22	15.7	16.3– 2.6	1.6– 0.2 (15.9%)	4.5–0.9 (19.4%)	1.5–0.3 (17.4%)
23	22.4	63.9–14.3	47.8–10.6 (22.2%)	6.4–2.4 (36.8%)	3.3–0.5 (16.6%)
24	38.0	29.1– 6.3	17.9– 6.9 (38.8%)	6.5–3.5 (53.9%)	6.5–2.3 (35.4%)
25	44.4	19.3– 8.6	54.6–36.0 (65.9%)	3.5–2.4 (67.9%)	17.3–9.4 (54.3%)
Mean	40.1	26.2– 8.3	22.6–10.1 (44.5%)	4.8–2.4 (48.6%)	5.6–2.4 (42.8%)
S.E.	7.6	8.2 2.2	9.3 5.4	0.7 0.5	2.5 1.4
p*	$p<0.001$	N.S. $p<0.01$	N.S. N.S.	$p<0.01$ N.S.	$p<0.01$ N.S.

* Significance of the means of the Table 3 are evaluated against the means in the Table 2. N.S. =not significant

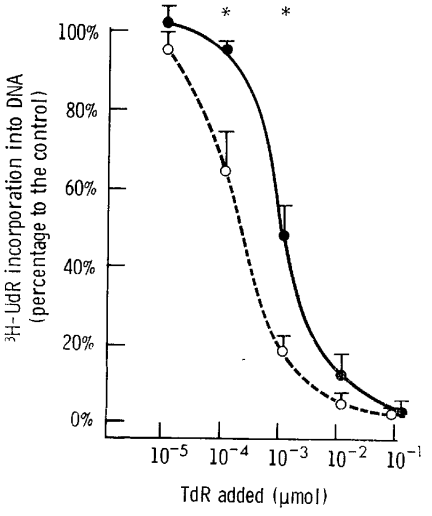

Fig. 2. Effect of thymidine on the uptake of ^{3}H-UdR into DNA of the normoblastic and megaloblastic bone marrow cells. Twenty four normoblastic and 15 megaloblastic marrows were examined. ●——●, megaloblastic marrow; ○- - -○, normoblastic marrow; upward bar indicates standard error of the mean. * $p<0.01$.

μmole/μg DNA and 20.0×10^{-7} μmole/10^6 cells to 2.4 and 3.0, respectively. In megaloblastic marrows, from 4.8×10^{-8} μmole/μg DNA and 5.6×10^{-7} μmole /10^6 cells to 2.4 and 2.4, respectively.

Although no difference of the initial ^{3}H-TdR incorporation was observed between normoblastic and megaloblastic marrows, ^{3}H-TdR uptake was significantly higher in megaloblastic marrows than in normoblastic ones after adding UdR. On the other hand, initial uptake of ^{3}H-UdR was significantly reduced in megaloblastic marrows, although ^{3}H-UdR uptake after an addition of TdR was the same level in both megaloblastic and normoblastic marrows.

DISCUSSION

The DUS test has been appreciated to be of great value for making the diagnosis of megaloblastic anemia (2, 6, 7). Although several modifications of the original Killman's method appeared (2, 3, 5, 6), no attempt has been made to compare the results obtained from different methods on the same patients. We modified slightly the method described by Wickramasinghe and Longland (6). They counted cell numbers of each tube after incubation. We counted cell numbers before delivering cells into tubes, and after that, the same volume of cell suspension was carefully pipetted into six tubes (three each for Control and UdR-added Test). A good correlation with that of Metz *et al.* (2) was obtained by the

modified method. Therefore, this simple method can be applied for a diagnostic tool of megaloblastic anemia. Normal range was 0.2–10%. All megaloblastic marrows showed values over 10%. Although we had one case of megaloblastic anemia showing 13.7% in DUS test, the bone marrow of the case was megaloblastic in very mild degree. Sive, Green and Metz (4) reported that nine normal marrows showed the levels ranging 2.8–11.9% by this test. The diagnosis of megaloblastic anemia should be avoided if the DUS test value falls between 10% and 15%. In the marrows of patients receiving anticonvulsants, discrepancies were reported between the DUS test and macrocytosis or megaloblastosis (8). According to the observations of Taguchi *et al.* (9), in four normoblastic marrows from the patients receiving anticonvulsants, DUS levels were between 10–15%. Another occasion in which DUS test is not reliable is the association of iron deficiency (10, 11). Patients may have a vitamin B_{12} or folate deficiency even if DUS test is below 15%, if iron deficiency is associated. Therefore, on those occasions, we should use another test or should repeat DUS test after iron therapy.

Killman (1) explained the abnormality of DUS test in megaloblastic marrow by a block of methylation of deoxyuridylate. On the other hand, Beck (12) proposed that UdR inhibited DNA synthesis *in vitro* and that in megaloblastic marrow cells, UdR could not suppress the incorporation of ^{3}H-TdR to the same extent as in normoblastic marrows because the uptake of ^{3}H-TdR without UdR was already low to begin with. However we could not confirm his result. Absolute amount of TdR incorporated into DNA was not reduced in megaloblastic marrows before the addition of UdR. Suppression of ^{3}H-TdR incorporation by UdR was actually less in megaloblastic marrows than in normoblastic ones. Although Wickramasinghe and Longland (11) reported that ^{3}H-TdR uptake per 10^3 DNA synthesizing cells was approximately half the mean value of the normal marrows, the calculation of DUS test is not based on the DNA synthesizing cells but on total cells or total DNA amount of the marrow cells. Therefore, it seems to be reasonable to conclude that addition of UdR reduces the incorporation of ^{3}H-TdR to a greater extent in normoblatic marrows than in megaloblastic ones.

In a reciprocal experiment, ^{3}H-UdR incorporation into DNA was lower in megaloblastic than in normoblastic marrows. This result can be easily explained by the present knowledge that the pathway from deoxyuridylate to thymidylate is inhibited in megaloblastic anemia. It is rather difficult to explain the fact that larger amount of TdR was necessary to suppress ^{3}H-UdR incorporation in megaloblastic than in normoblastic marrows. As mentioned previously, TdR uptake was not reduced in megaloblastic marrows. Although percentage suppression of ^{3}H-UdR uptake was lower in megaloblastic marrows, no difference was seen in absolute amount of ^{3}H-UdR uptake after preincubation with TdR. Therefore,

lower rate of suppression of ^{3}H-UdR by TdR in megaloblastic marrows may be due to low ^{3}H-UdR uptake before the addition of TdR, and calculated percentage in megaloblastic marrows showed higher levels than in normoblastic marrows.

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